IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

(Attorney Docket No. 01-660)

In re the Application of:		
	Mohammad S. Nasir and Michael E. Jolley	Group Art Unit: 1641
Serial No.:	09/905,452	Examiner: Deborah A. Davis
Filed:	July 13, 2001	
For:	Fluorescence Polarization-Based Homogeneous Assay for Aflatoxins	

APPEAL BRIEF

Richard A. Machonkin
McDONNELL BOEHNEN
HULBERT & BERGHOFF LLP
300 South Wacker Drive
Chicago, Illinois 60606
(312) 913-0001

Table of Contents

McDONNELL BOEHNEN
HULBERT & BERGHOFF LLP
300 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 913-0001

I. Real Party in Interest

The real party in interest is Diachemix LLC, to which this invention is assigned.

II. Related Appeals and Interferences

Applicant is not aware of any related appeals or interferences.

III. Status of Claims

Claims 1-18 are pending and stand rejected. The rejection of claims 1-18 is being appealed. A clean set of the pending claims is attached in the Claims Appendix beginning at page 12.

IV. Status of Amendments

No amendments were filed subsequent to the final rejection mailed August 24, 2005.

V. Summary of Claimed Subject Matter

Of claims 1-18, claims 1 and 11 are independent. Claims 2-10 are dependent on claim 1, and claims 12-18 are dependent on claim 11.

Claim 1 is directed to a homogeneous assay for the determination of aflatoxins in agricultural products. The method recited in claim 1 comprises the steps of: (i) extracting aflatoxin from a sample to provide an extract (*see* Specification, p. 10, lines 9-14); (ii) combining said extract with a tracer and an antibody to provide a mixture (*see* Specification, p. 10, lines 15-20), said tracer comprising an aflatoxin oxime conjugated to a fluorophore, said tracer being able to bind to said antibody to produce a detectable change in fluorescence polarization (*see* Specification, p. 6, line 15 – p. 8, line 13); (iii) measuring the fluorescence polarization of said mixture to obtain a measured fluorescence polarization (*see* Specification, p. 10, lines 20-21); and (iv) comparing said measured fluorescence polarization with a characterized fluorescence

McDONNELL BOEHNEN
HULBERT & BERGHOFF LLP
300 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 913-0001

polarization value, said characterized fluorescence polarization value corresponding to a known aflatoxin concentration (*see* Specification, p. 10, line 22 – p. 11, line 10).

Claim 11 is directed to an assay kit for the determination of aflatoxins in agricultural products in a homogeneous assay. The assay kit comprises an antibody and a tracer, each in an amount suitable for at least one assay, and suitable packaging, said tracer comprising an aflatoxin oxime conjugated to a fluorophore, said tracer being able to bind to said antibody to produce a detectable change in fluorescence polarization in a homogeneous assay (*see* Specification, p. 14, line 12 – p. 15, line 21).

VI. Grounds of Rejection to be Reviewed on Appeal

Claims 1-4, 8, and 11-18 stand rejected under 35 U.S.C. § 103 as being allegedly obvious over a combination of Dixon et al., U.S. Patent No. 4,835,100 ("Dixon") and Nasir et al., "Fluorescence Polarization: An analytical Tool for Immunoassay and Drug Discovery, Combinatorial Chemistry & High Throughput Screening, 1999, vol. 2, pp. 177-190 ("Nasir"). Claims 5-7 stand rejected under 35 U.S.C. § 103 as being allegedly obvious over a combination of Dixon, Nasir, and Michel et al., U.S. Patent No. 5,741,654 ("Michel"). Claims 9 and 10 stand rejected under 35 U.S.C. § 103 as being allegedly obvious over a combination of Dixon, Nasir, and McMahon et al., U.S. Patent No. 5,166,078 ("McMahon").

McDONNELL BOEHNEN
HULBERT & BERGHOFF LLP
300 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 913-0001

VII. Argument

A. The Examiner Erred in Rejecting Claims 1-4, 8, and 11-18 as Being Obvious over a Combination of Dixon and Nasir

The 35 U.S.C. § 103(a) rejections of the independent claims are improper, because the Examiner has failed to establish a *prima facie* case of obviousness of these claims over a combination of Dixon and Nasir. In order to establish a *prima facie* case of obviousness over a combination of references, the combination must teach or suggest all of the claim limitations. M.P.E.P. § 2143.03; *In re Royka*, 490 F.2d 981, 180 USPQ 580 (CCPA 1974). In this case, however, even if Dixon and Nair were to be combined together, the resulting combination still fails to teach or suggest all of the limitations recited in claims 1-4, 8, and 11-18. In particular, the Dixon/Nasir combination fails to teach or suggest either of the following:

- a "tracer comprising an aflatoxin oxime conjugated to a fluorophore," as recited in independent claims 1 and 11; and
- "said tracer being able to bind to said antibody to produce a detectable change in fluorescence polarization," as recited in claims 1 and 11.

Because the Examiner has failed to establish even a *prima facie* case of obviousness, the Examiner has erred as a matter of law.

1. Neither Dixon nor Nasir teach or suggest a tracer comprising an aflatoxin oxime conjugated to a fluorophore

Independent claims 1 and 11 both recite a "tracer comprising an aflatoxin oxime conjugated to a fluorophore." However, neither Dixon nor Nasir teach or suggest such a tracer. As set forth below, Dixon actually teaches a tracer comprising an aflatoxin oxime conjugated to a ***protein***, not a ***fluorophore***. In addition, Nasir does not specifically refer to aflatoxin at all.

McDONNELL BOEHNEN
HULBERT & BERGHOFF LLP
300 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 913-0001

Moreover, to the extent that Nasir teaches or suggests anything about aflatoxins, it would be to conjugate aflatoxin itself, rather than aflatoxin oxime, to a fluorophore. Therefore, Nasir actually teaches away from a tracer comprising an aflatoxin oxime conjugated to a fluorophore.

Because neither reference in the Examiner's combination teaches or suggests an aflatoxin oxime conjugated to fluorophore, the Examiner has clearly erred in rejecting the pending claims.

a. Dixon teaches conjugating an aflatoxin oxime to a protein, not to a fluorophore

The Examiner has argued that Dixon teaches aflatoxins conjugated to a "label," and that the aflatoxin is converted to an oxime for labeling. *See* Final Office Action, pp. 2, 6-7. However, the "labels" in Dixon are proteins, not fluorophores.

In particular, Dixon refers to aflatoxin being conjugated to bovine serum albumin (BSA) for use as an immunogen (col. 4, lines 62-64), to ovalbumin (OA) for use as a solid phase antigen in competitive indirect ELISA (col. 4, lines 64-66), and to horseradish peroxidise (HRP) for use in competitive direct ELISA (col. 6, lines 34-37). However, of these, only the HRP is truly a label. The BSA is used to generate antibodies, not as a label in an assay (col. 5, lines 16-26). The OA is used in an ELISA assay, but the actual label in the ELISA assay is horseradish peroxidise (HRP). The HRP acts on a substrate (ABTS) to yield a change in absorbance at 405 nm (col. 5, lines 48-60). Thus, the only labelled aflatoxin in Dixon is the "aflatoxin B_1-HRP conjugate," which acts on the ABTS substrate in an ELISA assay (col. 6, lines 34-44).

Dixon does not state explicitly how the aflatoxin B_1-HRP conjugate is prepared. However, assuming that this conjugate is prepared by converting the aflatoxin to aflatoxin oxime, as Dixon describes for BSA and OA labelling, the fact remains that the HRP enzyme label is chemically and functionally very different from a fluorophore label. As noted above, the HRP

McDONNELL BOEHNEN
HULBERT & BERGHOFF LLP
300 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 913-0001

functions as a label by acting on a substrate (ABTS) in order to make the results of the assay visible (e.g., by measuring absorbance at 405 nm). In contrast, fluorophores do not function as labels by acting on substrates; rather, fluorophores emit photons of light when they are excited with photons of suitable energy (Nasir, p. 178). In addition, the aflatoxin B_1-HRP conjugate is used in a heterogeneous assay, namely, a solid-phase ELISA assay (col. 6, lines 23-44). In contrast, the fluorophore of claims 1 and 11 is used in a homogenous assay. These differences between the HRP label in Dixon and the claimed fluorophore establish that Dixon does not teach or suggest a "tracer comprising an aflatoxin oxime conjugated to a fluorophore," as recited in claim 1 and 11.

Furthermore, the Nasir reference in the Examiner's Dixon/Nasir combination does not make up for this deficiency in Dixon. Although Nasir discusses the use of fluorophores in fluorescence polarization assays, replacing the HRP label in Dixon with a fluorophore would change the principle of operation set forth in Dixon. As noted above, HRP labels and fluorophores function in very different ways (an HRP label acts on a substrate, whereas a fluorophore does not), and Dixon's HRP label is used in a heterogeneous assay, whereas the claimed fluorophore is used in a homogeneous assay. Because the Examiner's rationale under § 103(a), in which Dixon's HRP label in a heterogeneous assay is replaced with a fluorophore in a homogeneous assay, would improperly change the principle of operation set forth in Dixon, the Examiner has not established a *prima facie* case of obvious as a matter of law:

> If the proposed modification or combination of the prior art would change the principle of operation of the prior art invention being modified, then the teachings of the references are not sufficient to render the claims *prima facie* obvious.

M.P.E.P. § 2143.01(VI), citing *In re Ratti*, 270 F.2d 810, 123 USPQ 349 (CCPA 1959).

McDONNELL BOEHNEN
HULBERT & BERGHOFF LLP
300 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 913-0001

b. Nasir teaches away from an aflatoxin oxime conjugated to a fluorophore

The Examiner has argued that it would have been obvious to one of ordinary skill in the art to modify Dixon to detect aflatoxins using a fluorescence polarization assay as taught by Nasir. *See* Final Office Action, p. 3. However, Nasir does not specifically refer to aflatoxins at all. Instead, Nasir refers to mycotoxins, a class of toxins that includes aflatoxins but also includes many other toxins having a wide array of chemical structures (Pestka, p. 120). The reference to mycotoxins occurs in a section of Nasir titled "Detection of Toxins in Grains and Environmental Samples" (Nasir, pp. 181-182). Significantly, that section does not report any actual results for fluorescence polarization (FP) assays for mycotoxins. Instead, the section states that the FP format "is a technique of great potential in this area of research." By describing the potential for results, rather than actual results, Nasir does not teach a reasonable expectation of success with respect to a fluorescence polarization assay for aflatoxins or any other mycotoxins, as would be required to establish a *prima facie* case of obviousness. M.P.E.P. § 2143, citing *In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991).

Even if the statements in Nasir regarding mycotoxin research could be applied to aflatoxin specifically, the statements actually teach away from conjugating aflatoxin oxime with a fluorophore. In particular, Nasir states that the mycotoxin itself is labelled:

> A mycotoxin antigen of interest is labeled with a suitable fluorescent molecule (tracer).

(Nasir, p. 182, col. 1). One following this statement would probably try to label aflatoxin with a fluorophore. Indeed, this is what the inventors tried to do -- but they found that it did not work. (Nasir Declaration, ¶ 5). It was not until the inventors took the innovative approach of

McDONNELL BOEHNEN
HULBERT & BERGHOFF LLP
300 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 913-0001

conjugating a fluorophore to an aflatoxin oxime instead that they were able to develop a successful tracer. (Nasir Declaration, ¶ 6). Because the Nasir reference teaches labelling the mycotoxin itself, rather than an oxime or other derivative thereof, Nasir teaches away from an aflatoxin ***oxime*** conjugated to a fluorophore. The law is clear that it is improper to combine references where the references teach away from their combination. M.P.E.P. § 2145(X)(D)(2), citing *In re Graselli*, 713 F.2d 731, 743, 218 USPQ 769, 779 (Fed. Cir. 1993). Thus, the Examiner's attempt to combine Dixon with Nasir is improper.

The law is also clear that proceeding contrary to the accepted wisdom in the art is evidence of nonobvious. M.P.E.P. § 2145(X)(D)(3), citing *In re Hedges*, 783 F.2d 1038, 228 USPQ 685 (Fed. Cir. 1986). As noted above, Nasir states that the mycotoxin itself, not a derivative, is conjugated to a fluorophore. The Examiner has not identified anything in the prior art that contradicts this accepted wisdom. Although Dixon states that "[s]ince aflatoxin B_1 possesses no reactive groups for conjugation, it was first converted to aflatoxin-B_1-carboxymethylamine (aflatoxin B_1-oxime) ..." this statement refers to conjugating aflatoxin to ***proteins***, i.e., BSA and OA (col. 4, line 62 – col. 5, line 5). The statement does not imply that conversion to an oxime is necessary in order to conjugate ***fluorophores***. Accordingly, the approach taken in the claimed invention of conjugating an aflatoxin oxime to a fluorophore is contrary to the teachings of Nasir and, therefore, nonobvious.

2. The prior art does not teach a tracer, comprising an aflatoxin oxime conjugated to a fluorophore, being able to bind to an antibody specific for aflatoxin to produce a detectable change in fluorescence polarization

The Examiner has failed utterly to identify any prior art teaching of a tracer, comprising an aflatoxin oxime conjugated to a fluorophore, that has the special property of being able to bind

McDONNELL BOEHNEN
HULBERT & BERGHOFF LLP
300 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 913-0001

to an antibody specific to aflatoxin to produce a detectable change in fluorescence polarization, as recited in claims 1 and 11. Dixon does not refer to fluorescence polarization at all. Nasir does not specifically refer to aflatoxins at all. Moreover, Nasir describes fluorescence polarization as simply having "great potential" for mycotoxins, without reporting any results of any successful FP assay for aflatoxins or any other mycotoxins. Thus, the Dixon/Nasir combination falls far short of teaching a tracer that has the special property of being able to bind to an antibody to produce a detectable change in fluorescence polarization, as claimed in claims 1 and 11.

More particularly, the Examiner has not identified any prior art teaching that an aflatoxin oxime conjugated to a fluorophore would still be able to bind to an antibody specific for aflatoxin. Although Dixon teaches that aflatoxin oxime conjugated a ***protein*** (i.e., BSA, OA, and HRP) is able to bind to antibodies specific to aflatoxin in a heterogeneous ELISA assay, it does not follow that an aflatoxin oxime conjugated to a ***fluorophore*** would be able to bind to specific antibodies in a homogeneous fluorescence polarization assay. As noted in the Nasir reference, "[i]n ELISA compounds adsorbed to the solid phase may have different affinites than in solution." (Nasir, p. 181, col. 2).

The Examiner has also failed to identify any prior art teaching that if binding occurs it would produce a detectable change in fluorescence polarization. In particular, it is by no means inevitable that binding will actually result in a detectable change in fluorescence polarization. The Nasir reference describes a phenomenon called the "propeller effect," whereby "although binding has occurred, little polarization shift is observed" (Nasir, p. 180, col. 2). The Nasir reference further explains that the effect is "caused by the uncoupling of the fluorophore and binding site due to a long, flexible linkage" and that "[t]o attain the best results one must employ

McDONNELL BOEHNEN
HULBERT & BERGHOFF LLP
300 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 913-0001

the shortest and most rigid linkage possible between the fluorophore and the ligand." Because the Nasir reference does not describe any results for aflatoxin, Nasir does not teach one of ordinary skill in the art whether an aflatoxin-based tracer would actually produce a detectable change in fluorescence polarization upon binding to a specific antibody or whether it would suffer from the "propeller effect."

Moreover, the claimed tracer is not simply an aflatoxin-based tracer but a tracer that comprises an aflatoxin ***oxime*** conjugated to a fluorophore. The addition of an oxime linkage goes against Nasir's teaching of making the linkage between the fluorophore and the ligand as short and as rigid as possible. Thus, it is hardly surprising that the inventors did not know whether the aflatoxin oxime tracer would be able to bind to the antibody to produce a detectable change in fluorescence polarization until the tracer was actually tested. (Nasir Declaration, ¶ 6). Indeed, for many fluorophores that were tested, there was no appreciable change in polarization values upon reaction to the antibody solution. (Nasir Declaration, ¶ 6).

Finally, because of the sheer number and variety of mycotoxins, Nasir's general statements regarding "mycotoxins" do not suggest the successful application of the fluorescence polarization technique to aflatoxins specifically. In addition to aflatoxins, the class of mycotoxins includes cyclopiazonic acid, ergot alkaloid, fumonisins, fusarochromanone, ochratoxin, PR toxin, rubratoxin, sterigmatocystin, trichothecenes (acetyldeoxynivalenol, diacetoxyscirpenol, nivalenol, roridin A, and T-2 toxin), and zearalenone. (Pestka, p. 125). Moreover, "[t]hese compounds have a wide array of chemical structures." (Pestka, p. 120, col. 1). Given this variety in chemical structures, and the fact that the "propeller effect" can result in little or no observed polarization shift, one of ordinary skill in the art would not understand from

McDONNELL BOEHNEN
HULBERT & BERGHOFF LLP
300 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 913-0001

Nasir's statements that the fluorescence polarization technique would necessarily work to detect any and all mycotoxins. Instead, what the skilled artisan would understand from Nasir's statement that FP has "great potential" for mycotoxins is that further research was required. Thus, the Examiner's rejection is based on an improper "obvious to try" rationale to "explore a new technology or general approach that seemed to be a promising field of experimentation, where the prior art gave only general guidance as to the particular form of the claimed invention or how to achieve it." M.P.E.P. § 2145(X)(B), citing *In re O'Farrell*, 853 F.2d 894, 903, 7 USPQ2d 1673, 1681 (Fed. Cir. 1988).

In the face of this evidence, it is clear that the Examiner has not shown a prior art teaching that an aflatoxin-oxime based tracer would have the special property of being able to bind to antibodies specific to aflatoxin to produce a detectable change in fluorescence polarization.

B. The Examiner Erred in Rejecting Claims 5-7 as Being Obvious Over a Combination of Dixon, Nasir and Michel

Claims 5-7 depend from claim 1. As discussed above, the combination of Dixon and Nasir fails to teach or suggest all of the limitations of claim 1. Michel does not make up for the deficiencies in the Dixon/Nasir combination. Moreover, if an independent claim is nonobvious, then any claim depending therefrom is nonobvious. M.P.E.P. § 2143.03, citing *In re Fine*, 837 F.2d 1071, 5 USPQ2d 1596 (Fed. Cir. 1988). Thus, the Examiner's rejections of claim 5-7 are improper for at least the same reasons that the Examiner's rejection of claim 1 is improper.

C. The Examiner Erred in Rejecting Claims 9-10 as Being Obvious Over a Combination of Dixon, Nasir and McMahon

Claims 9-10 depend from claim 1. As discussed above, the combination of Dixon and Nasir fails to teach or suggest all of the limitations of claim 1. McMahon does not make up for

McDONNELL BOEHNEN
HULBERT & BERGHOFF LLP
300 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 913-0001

the deficiencies in the Dixon/Nasir combination. Moreover, if an independent claim is nonobvious, then any claim depending therefrom is nonobvious. M.P.E.P. § 2143.03, citing *In re Fine*, 837 F.2d 1071, 5 USPQ2d 1596 (Fed. Cir. 1988). Thus, the Examiner's rejections of claim 5-7 are improper for at least the same reasons that the Examiner's rejection of claim 1 is improper.

D. Conclusion

Applicant has demonstrated that the rejections of claims 1-18 are in error as a matter of law. Neither Dixon nor Nasir in the Examiner's Dixon/Nasir combination teaches a tracer comprising an aflatoxin oxime conjugated to a fluorophore. Moreover, even if the Dixon/Nasir combination somehow taught such a tracer, the Dixon/Nasir combination does not teach that the tracer would have the special property of being able to bind to antibodies specific to aflatoxin to produce a detectable change in fluorescence polarization.

Applicant therefore requests reversal of the rejections and allowance of all pending claims in this application.

Respectfully submitted,

MCDONNELL BOEHNEN
HULBERT & BERGHOFF LLP

Date: January 30, 2006

By: Richard A. Machonkin
Richard A. Machonkin
Reg. No. 41,962

McDONNELL BOEHNEN
HULBERT & BERGHOFF LLP
300 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 913-0001

VIII. CLAIMS APPENDIX

1. (original) A homogeneous assay for the determination of aflatoxins in agricultural products, said homogeneous assay comprising the steps of:

extracting aflatoxin from a sample to provide an extract;

combining said extract with a tracer and an antibody to provide a mixture, said antibody being specific for aflatoxin, said tracer comprising an aflatoxin oxime conjugated to a fluorophore, said tracer being able to bind to said antibody to produce a detectable change in fluorescence polarization;

measuring the fluorescence polarization of said mixture to obtain a measured fluorescence polarization; and

comparing said measured fluorescence polarization with a characterized fluorescence polarization value, said characterized fluorescence polarization value corresponding to a known aflatoxin concentration.

2. (original) The assay of claim 1, wherein said step of extracting aflatoxin from a sample to provide an extract comprises the steps of:

crushing said sample to provide a crushed sample; and

shaking said crushed sample with an extraction solvent for a predetermined time.

3. (original) The assay of claim 2, wherein said extraction solvent comprises an organic solvent and water.

McDONNELL BOEHNEN
HULBERT & BERGHOFF LLP
300 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 913-0001

4. (original) The assay of claim 3, wherein said organic solvent is methanol.

5. (original) The assay of claim 1, wherein said fluorophore is selected from the group consisting of fluoresceinamine, 5-aminoacetyl-amidofluorescein, and 5-(5-aminopentyl)-thioureidyl fluorescein.

6. (original) The assay of claim 5, wherein said fluorophore is an isomer of fluoresceinamine.

7. (original) The assay of claim 6, wherein said fluorophore is isomer 2 of fluoresceinamine.

8. (original) The assay of claim 1, wherein said aflatoxin oxime is (Aflatoxin B_1)-O-carboxymethyloxime.

9. (original) The assay of claim 1, further comprising the steps of:

providing a plurality of aflatoxin standard solutions, each of said aflatoxin standard solutions having a different known concentration of aflatoxin;

adding said tracer and said antibody to each one of said plurality of aflatoxin standard solutions, so as to provide a plurality of standard mixtures; and

McDONNELL BOEHNEN
HULBERT & BERGHOFF LLP
300 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 913-0001

measuring the fluorescence polarization of each one of said plurality of said standard mixtures to provide a plurality of standard fluorescence polarization values corresponding to known aflatoxin concentrations.

10. (original) The assay of claim 9, wherein said characterized fluorescence polarization value is one of said standard fluorescence polarization values.

11. (previously presented) An assay kit for the determination of aflatoxins in agricultural products in a homogeneous assay, said assay kit comprising:

an antibody and a tracer, each in an amount suitable for at least one assay, and suitable packaging, said antibody being specific for aflatoxin, said tracer comprising an aflatoxin oxime conjugated to a fluorophore, said tracer being able to bind to said antibody to produce a detectable change in fluorescence polarization in a homogeneous assay.

12. (original) The assay kit of claim 11, further comprising an extraction solvent for extracting aflatoxin from a sample.

13. (original) The assay kit of claim 12, wherein said extraction solvent comprises an organic solvent and water.

14. (original) The assay kit of claim 13, wherein said organic solvent is methanol.

McDONNELL BOEHNEN
HULBERT & BERGHOFF LLP
300 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 913-0001

15. (original) The assay kit of claim 11, wherein said fluorophore is selected from the group consisting of fluoresceinamine, 5-aminoacetyl-amidofluorescein, and 5-(5-aminopentyl)-thioureidyl fluorescein.

16. (original) The assay kit of claim 15, wherein said fluorophore is fluoresceinamine.

17. (original) The assay kit of claim 16, wherein said fluorophore is isomer 2 of fluoresceinamine.

18. (original) The assay kit of claim 11, wherein said aflatoxin oxime is (Aflatoxin B_1)-O-carboxymethyloxime.

McDONNELL BOEHNEN
HULBERT & BERGHOFF LLP
300 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 913-0001

IX. EVIDENCE APPENDIX

This appendix contains copies of the following evidence:

1. Declaration of Mohammad S. Nasir Pursuant to 37 C.F.R. § 1.132
2. Pestka et al., "Immunological Assays for Mycotoxin Detection," Food Technology, February 1995, pp. 120-128

This evidence was submitted with a Response filed June 8, 2004 and entered in the record by the Examiner.

McDONNELL BOEHNEN
HULBERT & BERGHOFF LLP
300 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 913-0001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
(Case No. 01-660)

In the Application of:	)	
	)	
Mohammad S. Nasir and Michael E. Jolley	)	Examiner: Deborah A. Davis
	)	
Serial No.: 09/905,452	)	Art Unit: 1641
	)	
Filed: July 13, 2001	)	
	)	
For: Fluorescence Polarization-Based Homogeneous Assay for Aflatoxins	)	

DECLARATION OF MOHAMMAD S. NASIR PURSUANT TO 37 C.F.R. § 1.132

I, Mohammad S. Nasir, hereby declares as follows:

1. I am a principal scientist at Diachemix LLC, 683 E. Center Street, Suite H, Grayslake, Illinois 60030. I have held that position since 1997.

2. I received a PhD in chemistry from the State University of New York at Albany in 1990.

3. I am one of the inventors for the above-referenced patent application, and I am familiar with the research reported in the specification.

4. I, and my co-inventor, Michael E. Jolley, are the authors of the article "Fluorescence Polarization: An Analytical Tool for Immunoassay and Drug Discovery," *Combinatorial Chemistry & High Throughput Screening*, vol. 2, pp. 177-190 (1999).

5. Part of the research effort to develop a fluorescence polarization-based assay for aflatoxins involved attempts to label aflatoxin with a fluorophore, in order to develop a tracer that could be used in a fluorescence polarization assay for aflatoxins. However, the attempts to label aflatoxin with a fluorophore failed.

6. We were unable to develop a tracer until we found that aflatoxin oxime, a derivate of aflatoxin, could be labeled with a fluorophore. Even then, we did not know whether this tracer would be able to bind to the antibody to produce a detectable change in fluorescence polarization until we tested it. And many other fluorophores did not give any appreciable change in polarization values upon reaction to the antibody solution.

7. I hereby declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 5/26/04

Signed: Mohammad Nasir
Mohammad S. Nasir

A PUBLICATION OF THE
INSTITUTE OF FOOD TECHNOLOGISTS
THE SOCIETY FOR FOOD SCIENCE AND TECHNOLOGY
FEBRUARY 1995 • VOL. 49, NO. 2

FOOD TECHNOLOGY

p. 53

Special Report

Features

Papers followed by the symbol ⊛ have been peer reviewed.

Departments

Columns

Immunological Assays for Mycotoxin Detection

Enzyme-linked immunosorbent assays have been successfully applied to the screening of mycotoxins in a diverse array of foods

James J. Pestka, Mohamed N. Abouzied, and Sutikno

□ MYCOTOXINS ARE TOXIC SECondary metabolites produced by molds that often contaminate agricultural staples such as corn, wheat, and peanuts prior to harvest and during storage. These compounds have a wide array of chemical structures and are produced by common field and storage fungi, including many species of *Aspergillus, Penicillium,* and *Fusarium.*

Mycotoxins can elicit a variety of toxic symptoms in humans and animals, ranging from gastroenteritis to cancer (Table 1). For example, the aflatoxins were identified in the early 1960s as etiologic agents of hepatotoxicity and hepatic cancer in turkey poults and rainbow trout, respectively (Pestka and Casale, 1990). Strict regulations were subsequently established for aflatoxins in food in many countries because of the potential for similar effects in humans.

Table 1—**Major Mycotoxins and their Toxic Effects** *in experimental animal models*

Mycotoxin	Toxic effect	Commodity
Aflatoxins B_1, B_2, G_1, G_2	Liver toxicity and cancer	Corn, cottonseed, nuts, peanuts
Aflatoxin M_1	Liver toxicity and cancer	Milk
Cyclopiazonic acid	Muscle necrosis, oral lesions	Corn, peanuts
Fumonisin	Leukoencepholomacia, pulmonary edema	Corn
Ochratoxin	Kidney toxicity, cancer	Wheat, barley, corn, oats
Patulin	Generalized toxicity, neurotoxicity	Apples and apple juice
Trichothecenes, including deoxynivalenol (vomitoxin), nivalenol, T-2 toxin	Feed refusal, diarrhea, oral and GI lesions, immunotoxicity	Corn, wheat, barley
Zearalenone	Estrogenic effects, reproductive problems	Corn, wheat, barley

Besides direct concerns over human health, aflatoxins and other mycotoxins have major economic impact on livestock productivity as a result of lower quantity and quality of animal products, smaller litters, infertility, reduced feed efficiency, impaired resistance to disease, and loss of vaccination efficiency (CAST, 1989). Other economic effects can be more subtle but have major implications worldwide with regard to food production and processing (Table 2). It has been estimated by the Food and Agriculture Organization that 25% of the world's crops are affected by mycotoxins (Mannon and Johnson, 1985), although the absolute quantification of these losses remains enigmatic (Hesseltine, 1986).

Whether a mycotoxin is present in a food is largely dictated by environmental and biological factors (Fig. 1), particularly the regional weather during a growing season and harvest. A major means of eliminating mycotoxins from human and animal food is to detect and divert contaminated raw materials from feed and finished food use. Analytical surveillance can also assist in identifying those geographical regions where mycotoxins are a recurrent problem and provide a database for human exposure in epidemiological studies.

Methods for mycotoxin analysis include thin-layer, liquid, and gas chromatography, as well as mass spectroscopy. A major impediment to the implementation of these approaches is interferences found in extracts of corn, peanuts, wheat, cottonseed, and other foods as well as clinical samples from humans and animals. A series of extensive cleanup steps involving liquid–liquid partition, column cleanup, and evaporation are therefore required to overcome these interferences. The procedures are time-consuming and costly and often involve use of harmful solvents.

Research initiated in the late 1970s proposed the application of immunochemical assay, procedures commonly used in clinical laboratories, to the analysis of aflatoxin B_1 (AFB_1) and other mycotoxins (Chu and Ueno, 1977; Langone and Van Vunakis, 1976). These assays typically involve the competition between a free mycotoxin in a sample extract and a labeled mycotoxin for an antibody binding site. Although initially based on radioimmunoassay (RIA), subsequent research has established the feasibility of using enzyme-linked immunosorbent assay (ELISA). These and other assays have been made available commercially for the rapid assay of mycotoxins in food.

Key considerations in the development and application of mycotoxin immunoassays to food safety analysis include method of antibody generation, immunoassay formats, adaptability to food analysis, and evaluation criteria for commercial test kits.

Generation of Mycotoxin Antibodies

Antibodies, or immunoglobulins, are a family of glycoproteins that are produced as a response to foreign molecules in the body (Harlow and Lane, 1988). This response requires the interaction among white blood cells (leukocytes) known as B cells, T cells, and macrophage that culminate in the terminal differentiation of the B cells into antibody-secreting plasma cells.

Immunogenicity refers to the capacity of a macromolecule to induce an immune response and is dependent on its chemical structure and its ability to be recognized as foreign material. The minimum molecular weight of an immunogen is 3,000–5,000. Because mycotoxins are of low molecular weight (300–400), they must be conjugated to a carrier

Author Pestka, a Professional Member of IFT, is Professor, and authors Abouzied and Sutikno are Research Associate and Graduate Student, respectively, Dept. of Food Science and Human Nutrition, Michigan State University, East Lansing, MI 48824. Send reprint requests to author Pestka.

Table 2—**Economic Effects of Mycotoxin** *contamination in food. Modified from CAST (1989)*

Affected group	Economic effect
Producer	Food/feed loss
	Less income
	Possible loss of outlet
	Reduced livestock productivity
Handler/distributor	Less income
	Increased storage costs
	Market loss
	Litigation costs
Processor	Reduced-value products
	Insurance premiums
	Litigation costs
	Lower nutritional quality
	Increased product prices
	Possible chronic health effects
International trade	Unreliable supply, resulting in price fluctuation and market loss
	Problems with establishing production quotas and food security agreements

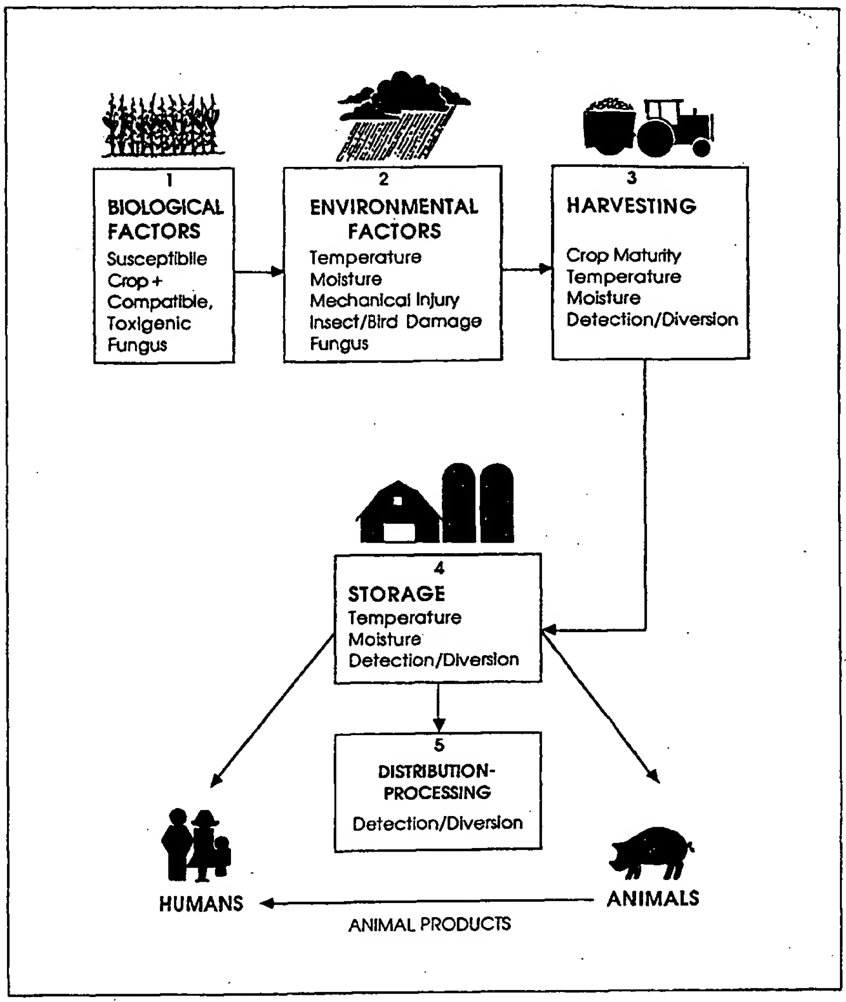


Fig. 1—**Factors Affecting Mycotoxin Occurrence** *in the food chain. From Pestka and Casale (1990)*

protein such as bovine serum albumin to be immunogenic.

Often, preparation of a suitable mycotoxin immunogen is the rate-limiting step in the development of an immunoassay. Standard hapten conjugation techniques used for mycotoxins have been reviewed by Chu (1986). If the mycotoxin doesn't have a reactive group for conjugation, it must be derivatized. For example, generation of protein conjugate for the mycotoxin fumonisin, (Azcona-Olivera et al., 1992a, b) simply involves the use of glutaraldehyde linkage via a free amino group (Fig. 2A), whereas conjugation of deoxynivalenol (Casale et al., 1988) is much more difficult because it involves extensive modification and blocking stages (Fig. 2B). Generally, the same conjugation techniques used for immunogen preparation can be applied to link mycotoxins to enzyme markers for the ELISA as long as the reaction conditions do not denature the enzyme.

In some cases, undesirable side reactions can occur during chemical conjugation and can result in antibodies to the by-products (Gendloff et al., 1986). Antibodies may also react with mycotoxin plus bridge groups, bridge group plus carrier protein, or the carrier protein directly. Thus, when characterizing and evaluating mycotoxin antibodies for immunoassay, conjugation reaction protocols must be carefully selected and appropriate controls utilized.

A key aspect in antibody development is the site of chemical conjugation. For example, many approaches have been used to produce antibodies with different specificities for the aflatoxin family (Fig. 3). Those portions of the aflatoxin molecule which project distally from the conjugation site are said to be *immunodominant*, because the resultant antibodies will exhibit the highest degree of recognition for these moieties. Thus, any metabolic precursor or analogues which mimic this immunodominant region will be recognized by an antibody generated against the parent toxin.

Cross-reactivity can be assessed using RIA or ELISA competition curves, where the levels required for 50% inhibition of marker ligand binding are used as the basis of comparison (Fig. 4). Rarely are these competition curves superimposable; thus, an analogue typically cross-reacts to a greater or lesser extent than the parent toxin. Although the presence of such analogues in a sample may render a quantitative assay to the level of semiquantitative, a high level of cross-reactivity can be very useful, as has been observed for the screening of fumonisins (Azcona-Olivera et al., 1992a, b), zearalenones (Dixon et al., 1987; Warner and Pestka, 1986) and the aflatoxins (Dixon et al., 1988).

The most straightforward approach to generation of antibodies is the multiple-site immunization of rabbits with 100–1,000 μg of mycotoxin–protein conjugate. Usable antiserum could be obtained in 3–4 mo. A critical factor in this immunization process is the use of an oil-based adjuvant containing killed *Mycobacterium* such as the "Freund's Complete type" to allow slow release of the immunogen and nonspecifically stimulate the immune response. More recently, we have successfully utilized very low levels of cholera toxin conjugates of fumonisin (Azcona-Olivera et al., 1992a, b) and a trichothecene mycotoxin (Abouzied et al., 1993). Although the mechanism(s) by which choleratoxin (CT) exerts its potent adjuvant effect in the immune system is not fully understood, it has been shown that CT concomitantly stimulates interleukin-1 production and antigen presentation (Bro-

mander et al., 1991).

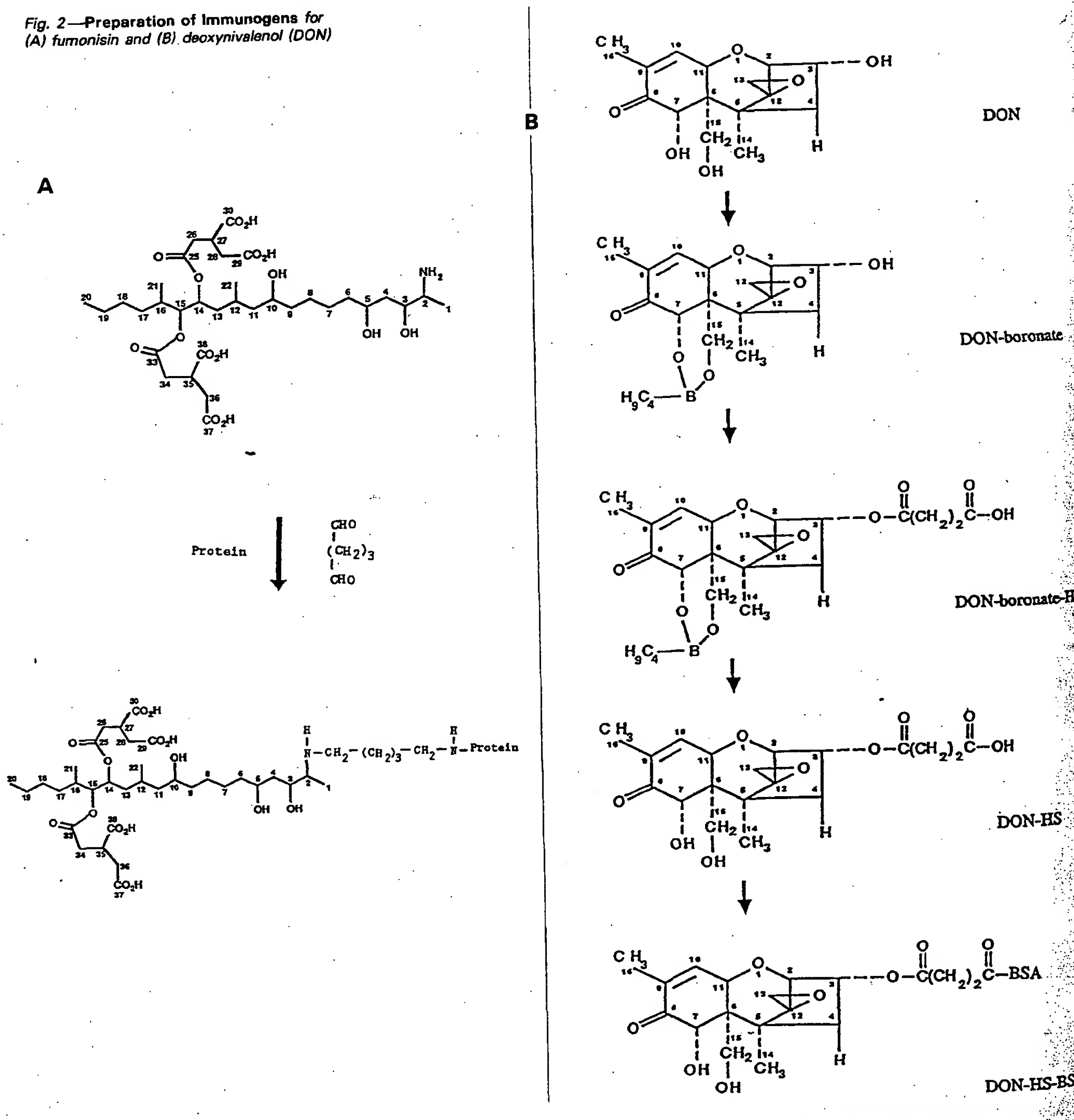
The advantages to this approach are severalfold. First, the procedure is rapid and yields quality antibodies, in comparison to poorer results achieved by standard protocols. Second, since no animal health impairment was observed at the concentrations used in this work, CT might be a humane alternative to Freund's adjuvant, which typically gives rise to abscesses, ulcers, or granulomas at the injection site. And third, the use of CT is also valuable when mycotoxin availability is limited, since relatively low doses of immunogen are required to induce a rapid and strong antibody response.

Rabbit antisera contain antibodies generated by multiple B-cell clones. These vary in specificity and are considered *polyclonal*. A major advantage of polyclonal antisera are that they are of high affinity and inexpensive to produce. However, inherent variability from lot to lot makes it difficult to use them in commercial kits with defined performance characteristics. Hybridomas have therefore been developed by fusions of immunized mouse spleen cells with a myeloma cell line to secrete reagent-quality *monoclonal* antibodies. A major disadvantage to this approach is the requirement for a tissue-culture facility, high cost, and time effort involved.

Mycotoxin Immunoassay Formats

A number of immunoassay formats have been devised for mycotoxin analysis. Initially, competitive RIAs were used whereby specific antibody is incubated with a constant amount of radiolabeled

Fig. 2—**Preparation of Immunogens** *for (A) fumonisin and (B) deoxynivalenol (DON)*



toxin in the presence of standard or unknown sample and then various procedures are used to remove the toxin antibody complex from solution. The amount of toxin in a sample is inversely related to the amount of radiolabeled (unbound) toxin in solution. Because of inherent problems with radioactivity, competitive assays based on ELISA (Engvall and Perlman, 1971) were devised. Both direct and indirect assays (Fig. 5) have been applied to mycotoxin detection. Microtiter plates, beads, and Terasaki plates have been used as solid-phase support for ELISA (Pestka et al., 1980; Pestka and Chu, 1984). High-protein-binding polystyrene microtiter plates have been most widely used because they offer an extensive support technology, including removable strips, multiwell pipettes, automated washers, and spectrophotometers.

Membranes are an alternative solid phase that have been employed for yes–no or threshold tests in cups, cards, and dipsticks. We have successfully employed nitrocellulose membranes in a Computer-Assisted-Multianalyte Assay System (CAMAS) for fumonisins, aflatoxins, and zearalenones (Abouzied and Pestka, 1994). Monoclonal antibodies for each of these toxins are immobilized as multiple lines on nitrocellulose membrane strips and sectored into hydrophobic compartments to minimize use of reagents. A modified ELISA is conducted whereby free mycotoxins and horseradish peroxidase–labeled mycotoxins compete for binding to the nitrocellulose-bound antibodies. Color intensity of lines formed by a precipitating substrate is inversely related to myco-

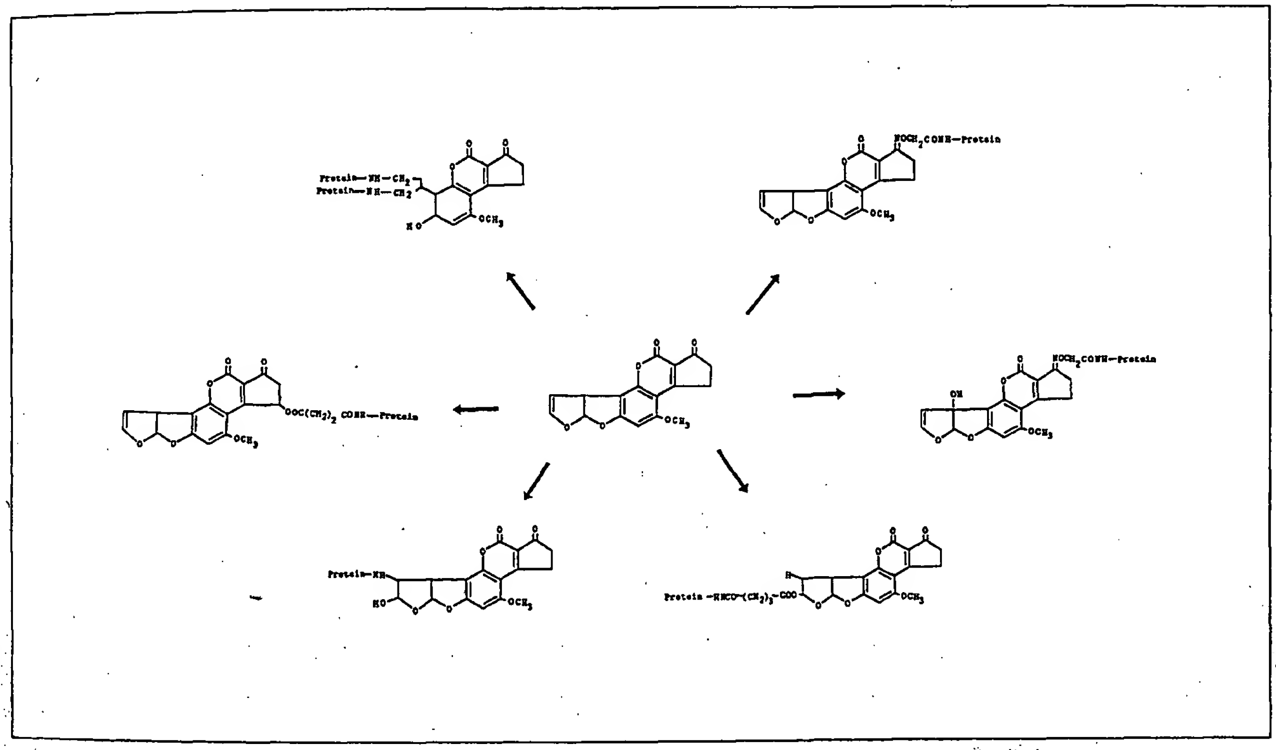


Fig. 3—**Approaches for Generation** *of aflatoxin immunogens*

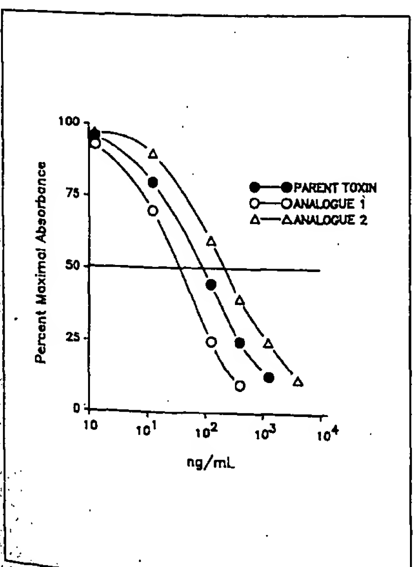
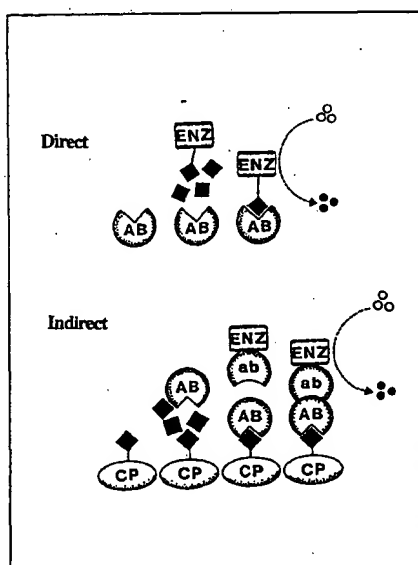


Fig. 4 (left)—**Theoretical Binding Inhibition Curves** *for mycotoxins in a competitive immunoassay. Cross-reactivity of analogue 1 is greater than for parent toxin, while that of analogue 2 is less*

Fig. 5 (right)—**Competitive ELISAs for Mycotoxins.** *In direct competitive ELISA, mycotoxin-enzyme conjugate ENZ is simultaneously incubated with unconjugated toxin ◆ over solid-phase-bound antibody AB. Toxin concentration is inversely related to bound enzyme conjugate and thus can be calculated on development of end-product absorbance obtained after addition of enzyme substrate. In indirect competitive ELISA, mycotoxin-specific antibody AB competes with free toxin for binding to solid-phase mycotoxin-carrier protein CP conjugate. Second anti-immunoglobulin enzyme conjugate ab–ENZ is then required to determine total bound antibody. Toxin concentration is inversely related to bound enzyme conjugate*



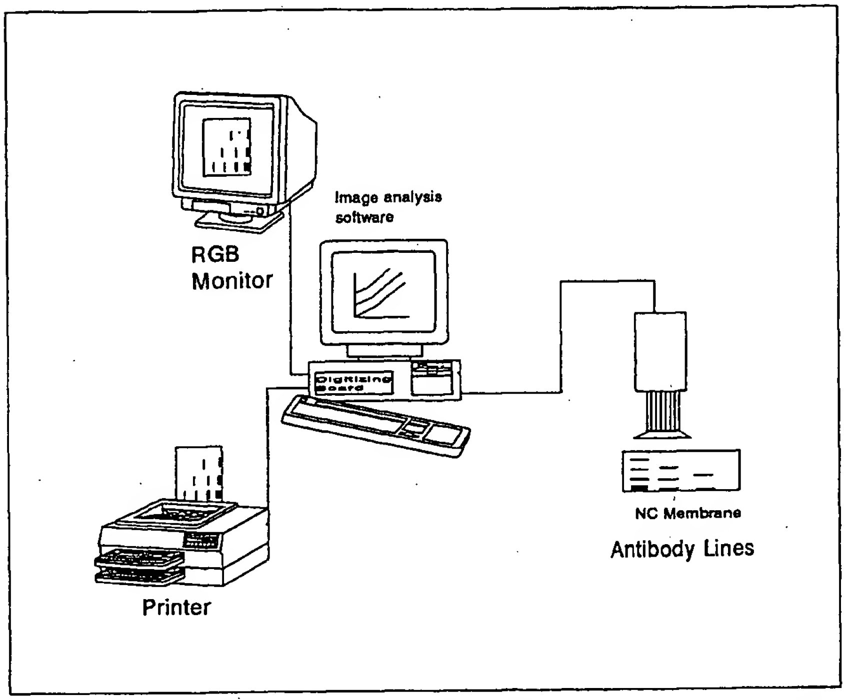


Fig. 6—**Image Analysis System** *used for Computer-Assisted Multianalyte System (CAMAS) for mycotoxins. See Abouzied and Pestka (1993) for details*

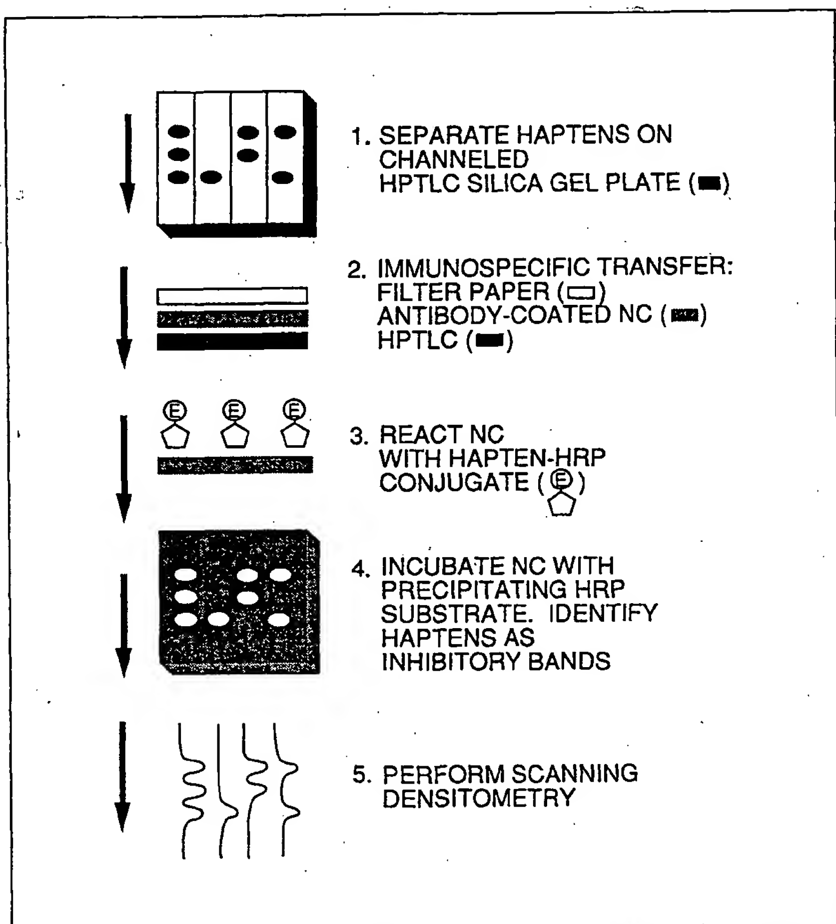


Fig. 7—**ELISAGRAM Procedure for Mycotoxins.** *From Pestka (1991)*

toxin concentration. Line density can be quantitatively assessed using a camera, video monitor, and microcomputer equipped with a video digitizing board (Fig. 6). The assay can be used to determine range values for the various mycotoxins in extracts of spiked corn in less than 30 min and record them on the microcomputer hard disk.

Another immunoblot approach, called ELISAGRAM, has been devised that combines the sensitivity and selectivity of competitive ELISA with the capacity of high-performance thin-layer chromatography (HPTLC) to separate structurally related mycotoxins (Pestka, 1991). The procedure (Fig. 7) involves separation of mycotoxin by HPTLC, blotting of the HPTLC plate with nitrocellulose (NC) coated with mycotoxin-specific monoclonal antibody, incubation of NC with mycotoxin-enzyme conjugate to identify unreacted antibody binding sites, detection of bound enzyme conjugate with a precipitating substrate, and visual or densitometric assessment of inhibition bands indicative of a cross-reacting mycotoxin. The technique has been applied to two major mycotoxin families, the zearalenones and aflatoxins. Multiple standard curves for the zearalenones and the aflatoxins can also be constructed using scanning densitometry. Cross-reactivity in ELISAGRAM curves are analogous to that found in competitive ELISA. This procedure could be widely applicable to the simultaneous quantitation and confirmation of multiple haptens with a single cross-reactive antibody.

Mycotoxin antibodies have also been attached to affinity columns and used for analytical purposes. For example, aflatoxins can be bound to an affinity column and then desorbed for subsequent derivatization and fluorescence measurement (Trucksess et al., 1991). This approach requires fluorescent mycotoxin derivatives and additional instrumentation (fluorometer). Another approach is to quantitate mycotoxin in the affinity column eluates by liquid chromatography (reviewed by Chu, 1992).

Immunoassay of Mycotoxins in Foods

Antibodies (polyclonal and monoclonal) have been made to the major known mycotoxins, and both ELISAs and RIAs have been successfully applied to the screening of mycotoxins in a diverse array of foods. Table 3 gives selected examples of reported immunoassays for the aflatoxins, ochratoxins, trichothecenes, fumonisins, and other mycotoxins, many of which can detect picogram or nanogram levels of toxin.

To maintain the native protein structure of the antibody and enzyme conjugate, immunoassays have to be carried out in an aqueous system. In a liquid system such as milk, mycotoxins can be analyzed directly (Pestka et al., 1981a), although the limit of detection can be increased by cleanup and concentration

—Text continued on page 127

Table 3—**Selected Immunological Assays for Mycotoxins in Foods**

Toxin	Format	Food analyzed	Limit of detection	Reference
Aflatoxin B_1	RIA	Corn, wheat, peanut butter	6 ng/g	El-Nakib et al. (1981)
	ELISA	Corn, wheat, peanut butter	3 ng/g	El-Nakib et al. (1981)
		Peanut butter	2.5 ng/g	Mortimer et al. (1988)
		Corn, cotton-seed	2.5 ng/g	Dixon et al. (1988)
		Barley	0.1 ng/mL	Ramakrishna et al. (1990)
Aflatoxins B_1 and G_1	ELISA	Peanut butter	0.3 ng/g	Morgan et al. (1986b)
Aflatoxin M_1	RIA	Milk	0.5 ng/g	Pestka et al. (1981b)
	ELISA	Milk	0.3 ng/g	Pestka et al. (1981b)
			0.3 ng/g	Fan et al. (1984)
			12 pg/g	Nieuwenhof et al. (1990)
	AC	Milk	50 pg/g	Hansen (1990)
Cyclopiazonic acid	ELISA	Buffer	30 pg/assay	Halnau and Weiler (1991)
Ergot alkaloid	ELISA	Wheat	10 ng/mL	Shelby and Kelley (1992)
Fumonisins	ELISA	Feed	250 ng/g	Azcona-Olivera et al. (1992a, b)
Fusarochromanone	LC-ELISA	Wheat, barley	5 ng/g	Yu and Chu (1991)
Ochratoxin	ELISA	Barley	1.0 ng/mL	Ramakrishna et al. (1990)
			5.0 ng/g	Candlish et al. (1988)
			0.1 ng/g	Morgan et al. (1983)
		Pig kidney	0.5 ng/g	Morgan et al. (1986a)
		Wheat, meat, plasma	1.0 ng/g	Sato et al. (1987)
		Chicken meat, wheat flour, porcine plasma, bovine sera	0.1 ng/mL	Kawamura et al. (1989, 1990)
PR Toxin	RIA	Cheese	50 μg/g	Wei and Chu (1988)
Rubratoxin	RIA	Cultures	0.1 μg	Davis and Stone (1979)
Sterigmatocystin	ELISA	Barley	0.01 pg	Morgan et al. (1986c)
Trichothecenes:				
Acetyldeoxynivalenol	ELISA	Rice	1 ng/g	Kemp et al. (1986)
Diacetoxyscirpenol	ELISA	Culture	16 ng/mL	Hack et al. (1989)
		Wheat	0.3 μg/mL	Mills et al. (1988)
Deoxynivalenol	RIA	Corn, wheat	20 ng/g	Xu et al. (1986)
	ELISA	Corn	200 ng/mL	Casale et al. (1988)
		Wheat	0.1 ng/assay	Mills et al. (1990)
		Grain-based food	1 μg/g	Abouzied et al. (1991)
Nivalenol	ELISA	Barley	0.1 ng/assay	Ikebuchi et al. (1990)
Roridin A	ELISA	Feed	5 ng/mL	Martlbauer et al. (1988)
T-2 toxin	RIA	Corn, wheat	0.1 ng/g	Lee and Chu (1981a)
		Milk	2.5 ng/g	Lee and Chu (1981b)
	ELISA	Corn	50 ng/g	Gendloff et al. (1984)
		Corn, wheat	2.5 ng/g	Pestka et al. (1981a)
		Milk	0.2 ng/g	Fan et al. (1984)
		Wheat	0.5 ng/g	Chiba et al. (1988)
Zearalenone	ELISA	Corn, wheat, feed	1 ng/mL	Liu et al. (1985)
		Corn	1 ng/g	Warner and Pestka (1986)
		Grain-based foods	2.5 ng/g	Warner and Pestka (1987)

Table 4—**Commercial Immunoassay Kits for Mycotoxins** *available as of June 1993*

Test kit	Analyte(s)	Format	Detection limit (ppb)	Analysis time[a]	Cost/assay ($)[b]	Application	Comments
EZ-Screen[c]	Aflatoxins	ELISA: Multi-site card	5-20	10	5.00-7.50	Corn, peanuts	Visual, pass/fail
	Aflatoxin M_1	ELISA: Multi-site card	0.5	10	5.00-7.50	Milk	Visual, pass/fail
	Ochratoxin	ELISA: Multi-site card	5	10	5.00-7.50	Corn	Visual, pass/fail
	T-2 toxin	ELISA: Multi-site card	12.5	10	5.00-7.50	Corn	Visual, pass/fail
	Zearalenone	ELISA: Multi-site card	50	10	5.00-7.50	Corn	Visual, pass/fail
Cite Probe[d]	Aflatoxin B_2	ELISA: Tray	5, 20	5	10.00	Corn, cottonseed	Visual, pass/fail, USDA-FGIS certified
Afla 5, 10, 20 Cup[e]	Aflatoxins	ELISA: Cup	5, 10, 20	5	4.00	Corn, peanuts, peanut butter, cottonseed, and feeds	Visual, pass/fail, USDA-FGIS certified, AOAC First Action Approval
One-Step ELISA[e]	Aflatoxin B_1	ELISA: Microwell	5	40	1.00	Corn, peanuts, peanut butter, cottonseed, and feeds	Quantitative; with ELISA reader
	Aflatoxin M_1	ELISA: Microwell	0.5	40	1.00	Milk	Quantitative; with ELISA reader
	Zearalenone	ELISA: Microwell	200	40	1.00	Corn, wheat	Quantitative; with ELISA reader
Agri-screen[f]	Aflatoxins	ELISA: Microwell	5	6-10	3.50	Corn, peanuts, cottonseed, feed	Visual or ELISA reader, pass/fail, USDA-FGIS certified, AOAC First Action Approval
	Deoxynivalenol (vomitoxin)	ELISA: Microwell	1,000	12-20	5.50	Corn, wheat, feed	Visual or ELISA reader, pass/fail, USDA-FGIS certified
	Fumonisin	ELISA: Microwell	500	12-20	5.50	Corn, wheat, feed	Visual or ELISA reader, pass/fail, USDA-FGIS certified
	Ochratoxin	ELISA: Microwell	20	12-20	5.75	Corn, wheat, feed	Visual or ELISA reader, pass/fail,
	T-2 toxin	ELISA: Microwell	500	18-30	5.50	Corn, wheat, feed	Visual or ELISA reader, pass/fail
	Zearalenone	ELISA: Microwell	250	18-30	5.50	Corn, wheat, feed	Visual or ELISA reader, pass/fail,
Veratox[f]	Aflatoxins	ELISA: Microwell	5	15	4.00	Corn, peanuts, cottonseed, feed	Quantitative with ELISA reader, USDA-FGIS certified
	Aflatoxin M_1	ELISA: Microwell	0.25	40	5.50	Milk	Quantitative with ELISA reader,
	Deoxynivalenol	ELISA: Microwell	300	20	5.50	Corn, wheat, feed	Quantitative with ELISA reader,
	T-2	ELISA: Microwell	50	30	6.00	Corn, wheat, feed	Quantitative with ELISA reader,
	Zearalenone	ELISA: Microwell	250	30	6.00	Corn, wheat, feed	Quantitative with ELISA reader,
Dosage[g]	Aflatoxin B_1	ELISA: Microwell	0.5	45	4.00	Corn, peanuts, feed	Quantitative with ELISA reader
Detection[g]	Aflatoxin B_1	ELISA: Tube	1, 5	45	9.00	Corn, peanuts, feed	Visual or reader, pass/fail
Aflatest[h]	Aflatoxins	Affinity column	1.0	10	7.00	Corn, peanuts, feed	Quantitative with fluorometer, USDA-FGIS certified, AOAC First Action Approval, IUPAC approval
	Aflatoxin M_1	Affinity column	0.1	10	10.00	Milk	Quantitative with fluorometer
Fumonitest[h]	Fumonisin B_1, B_2	Affinity column	2	15	10.00	Corn	Quantitative with fluorometer
Ochratest[h]	Ochratoxin	Affinity column	5	10	10.00	Corn	Quantitative with fluorometer
Zearaletest[h]	Zearalenone	Affinity column	500	10	10.00	Corn, wheat	Quantitative with fluorometer

[a]Analysis time does not include sample extraction
[b]Based on purchase of smallest available unit
[c]DiAGnostix, Inc., Burlington, NC 27215 (phone 800-334-1116)
[d]Idexx Labs, Westbrook, ME 04092 (800-548-6733)
[e]International Diagnostics, St. Joseph, MI 49085 (616-983-0972)
[f]Neogen Corp., Lansing, MI 48912 (800-234-5333)
[g]Transia, Lyon, France 69007 (33-72730381)
[h]Vicam, Somerville, MA 02145 (800-338-4381)

on a Sep-Pak or affinity column. Food samples were originally extracted with a solvent by standard protocols, evaporated, and reconstituted in an aqueous buffer for assay. However, based on our initial observation that mycotoxin–horseradish peroxidase and solid-phase antibodies retain sufficient stability for ELISA when incubated with as much as 35% (w/vol) methanol (Ram et al., 1986a), a direct approach was developed whereby solid substrates are blended with methanol–water extraction solvent and the extract analyzed directly or after dilution.

Immunoassay and various chromatographic methods for mycotoxin detection in foods are usually comparable when they are performed in the research laboratory (Ram et al., 1986a, b; Chu et al., 1987). However, sometimes toxin-free food extracts can interfere with mycotoxin–enzyme binding to the solid-phase antibody and therefore yield a low false-positive response when compared to a standard curve prepared in extraction solvent with buffer. Samples can be diluted more extensively to eliminate this interference, but this will decrease sensitivity. Alternatively, interference can be minimized by incorporating toxin-free sample extracts during standard-curve preparation. Another factor that must be considered is sample pH, which must sometimes be adjusted prior to immunoassay, since antibody–antigen binding occurs optimally at neutral pH.

Commercial Mycotoxin Immunoassays

The above research has led to the development of a number of commercial kits that have been marketed in the United States for food safety verification (Table 4). Commercial immunoassay kits have generally performed well in routine analyses performed in the laboratory and the field (Koeltzow and Tanner, 1990; Azer and Cooper, 1991; Dorner et al., 1993). However, when Horwitz et al. (1993) recalculated the precision performance parameters of collaborative studies for mycotoxins through 1991, they found that ELISA had somewhat poorer precision than thin-layer chromatography and liquid chromatography. Thus, when adopting a commercial immunoassay for rapid testing of mycotoxins, food analysts must critically evaluate the system in light of their specific needs and the limitations of the assay (Table 5).

Several organizations have provided leadership in evaluating these tests. Official First Action approval has been given for detection of aflatoxins by AOAC International (formerly Association of Official Analytical Chemists) in various commodities, using microtiter-well (Park et al., 1989a, b; Patey et al., 1992), cup (Trucksess et al., 1989), and affinity-column (Trucksess et al., 1991) tests. The U.S. Dept. of Agriculture's Federal Grain Inspection Service (FGIS) has tested and approved a number of kits for qualitative screening and quantitation of aflatoxins (Table 4).

Table 5—**Suggested Criteria** *for Adoption of a Mycotoxin Immunoassay. Summarized from Pestka (1988)*

Limits of detection and sensitivity range
Requirements for rapid screening and/or quantitation
Specificity
Necessity for adaptability to wide range of sample types
Effectiveness of recommended extraction procedure
Sample throughput
Field stability
Inter- and intra-assay reproducibility

To further facilitate kit evaluation, AOAC International has created the AOAC Research Institute with the charge of evaluating and certifying rapid test kits used for safety screening of foods. During summer 1993, this Institute evaluated aflatoxin test kits in conjunction with a Memorandum of Understanding signed in October 1992 with FGIS which recognizes the Test Kit Performance Testing Program for test kits that detect aflatoxins in grain. Upon successful evaluation using protocols by FGIS, test kits are certified to claim "Performance Tested in Accordance with Standards Established by FGIS for Test Kits to Detect Aflatoxin Residues in Grain and Grain Products." More recently, two deoxynivalenol ELISAs have been similarly certified by FGIS. It is anticipated that food analysts will be assisted by similar evaluation studies that focus on mycotoxins of potential health and economic significance, such as deoxynivalenol, the fumonisins, ochratoxins, and zearalenone.

References

Abouzied, M.M. and Pestka, J.J. 1994. Simultaneous screening of fumonisin B_1, aflatoxin B_1, and zearalenone by line immunoblot: A computer-assisted multanalyte assay system (CAMAS). J. AOAC Intl. 77: 495–501.

Abouzied, M.M., Azcona, J.I., Braselton, W.E., and Pestka, J.J. 1991. Immunochemical assessment of mycotoxins in 1989 grain foods: Evidence for deoxynivalenol (vomitoxin) contamination. Appl. Environ. Microbiol. 57: 672–677.

Azcona-Olivera, J.I., Abouzied, M.M., Plattner, R.D., and Pestka, J.J. 1992a. Production of monoclonal antibodies to the mycotoxins fumonisins B1, B2, and B3. J. Agric. Food Chem. 40: 531–534.

Azcona-Olivera, J.I., Abouzied, M.M., Plattner, R.D., Norred, W.P., and Pestka, J.J. 1992b. Generation of antibodies with fumonisins B1, B2, and B3. by using cholera toxin as the carrier-adjuvant. Appl. Environ. Microbiol. 58: 169–173.

Abouzied, M.M., Azcona-Olivera, J., Yoshizawa, T., and Pestka, J.J. 1993. Production of polyclonal antibodies to the trichothecene mycotoxin 4,15-diacetylnivalenol with the carrier-adjuvant cholera toxin. Appl. Environ. Microbiol. 59(5): 1264–1268.

Azer, M. and Cooper, C. 1991. Determination of aflatoxins in foods using HPLC and a commercial ELISA system. J. Food Protect. 54: 291–294.

Bromander, A., Holmgren, J., and Lycke, N. 1991. Cholera toxin stimulates IL-1 production and enhances antigen presentation by macrophages in vitro. J. Immunol. 146: 2908–2914.

Candlish, A.A.G., Stimson, W.H., and Smith, J.E. 1988. Determination of ochratoxin A by monoclonal antibody-based enzyme immunosorbent assay. J. Assn. Offic. Anal. Chem. 71: 961–964.

Casale, E.L., Pestka, J.J., and Hart, L.P. 1988. Enzyme-linked immunosorbent assay employing monoclonal antibody specific for deoxynivalenol (vomitoxin) and several analogues. J. Agric. Food Chem. 36: 663–668.

CAST. 1989. Mycotoxins—Economic and health risks. Task Force Report No. 116. Council for Agricultural Science and Technology, Ames, Iowa.

Chiba, J., Kawamura, O., Kaji, H., Ohtani, K., Nagayama, S., and Ueno, Y. 1988. A sensitive enzyme-linked immunosorbent assay for detection of T-2 toxin with monoclonal antibody. Food Addit. Contam. 5: 629–639.

Chu, F.S. 1986. Recent studies on immunochemical analysis of mycotoxins. In "Mycotoxins and Phycotoxins," 6th Intl. IUPAC Symp. on Mycotoxins and Phycotoxins, Pretoria, South Africa, July 22-25, 1985, ed. P. Steyn and R. Vleggaar, pp. 277–292.

Chu, F.S. 1992. Recent progress on analytical techniques for mycotoxins in feedstuffs. J. Animal Sci. 70: 3950–3963.

Chu, F.S. and Ueno, I. 1977. Production of antibody against aflatoxin B_1. Appl. Environ. Microbiol. 33: 1125–1128.

Chu, F.S., Fan, T.S.L., Zhang, G-S., and Xu, Y-C. 1987. Improved enzyme-linked immunosorbent assay for aflatoxin B_1 in agricultural commodities. J. Assn. Offic. Anal. Chem. 70: 854–857.

Davis, R.M. and Stone, S.S. 1979. Production of antirubratoxin antibody and its use in a radioimmunoassay for rubratoxin B. Mycopathology 67: 29–31.

Dixon, D.E., Ram, P.B., Hart, L.P., and Pestka, J.J. 1987. Hybridoma cell line production of a specific monoclonal antibody to the mycotoxins zearalenone and alpha-zearalenol. J. Agric. Food Chem. 35: 122–126.

Dixon, D.E., Pestka, J.J., Bidigare, B.A., Casale, W.L., Warner, R.L., Ram, B.P., and Hart, L.P. 1988. Production of sensitive monoclonal antibodies to aflatoxin B_1 and aflatoxin M_1 and their application to ELISA of naturally contaminated foods. J. Food Protect. 51: 201–204.

Dorner, J.W., Blankenship, P.D., and Cole, R.J. 1993. Performance of two immunochemical assays in the analysis of peanuts for aflatoxin at 37 field laboratories. J. AOAC Intl. 76: 637–643.

El-Nakib, O., Pestka, J.J., and Chu, F.S. 1981. Determination of aflatoxin B_1 in corn, wheat and peanut butter by enzyme-linked immunosorbent assay and solid phase radioimmunoassay. J Assn. Offic. Anal. Chem. 64: 1077–1082.

Engvall, E. and Perlman, P. 1971. Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of protein immunoglobulin G. Immunochemistry 8: 871–874.

Fan, T.S.L., Zhang, G.S., and Chu, F.S. 1984. An indirect enzyme-linked immunosorbent assay for T-2 toxin in biological fluids. J. Food Protect. 47: 964–968.

Gendloff, E.H., Pestka, J.J., Swanson, S.P., and Hart, L.P. 1984. Detection of T-2 toxin in *Fusarium sporotrichioides* infected corn by enzyme-linked immunosorbent assay. Appl. Environ. Microbiol. 47: 1161–1163.

—Continued on next page

Gendloff, E.H., Casale, W.L., Ram, B.P., Tai, J.H., Pestka, J.J., and Hart, L.P. 1986. Hapten-protein conjugates prepared by the mixed anhydride methods: Cross reactive antibodies in heterologous antisera. J. Immunol. Methodol. 92: 15–20.
Hack, R., Klaffer, U., and Terplan, G. 1989. A monoclonal antibody to the trichothecene mycotoxin diacetoxyscirpenol. Lett. Appl. Microbiol. 8: 71–76.
Halnau, S. and Weiler, E.W. 1991. Determination of the mycotoxin cyclopiazonic acid by enzyme immunoassay. J. Agric. Food Chem. 39: 1887–1891.
Hansen, J.J. 1990. Affinity column clean up and direct fluorescence measurement of aflatoxin M1 in raw milk. J. Food Protect. 53: 75–77.
Harlow, E. and Lane, D. 1988. Antibodies: A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
Hesseltine, C.W. 1986. Global significance of mycotoxins. In "Mycotoxins and Phycotoxins," 6th Intl. IUPAC Symp. on Mycotoxins and Phycotoxins, Pretoria, South Africa, July 22–25, 1985, ed. P.S. Steyn and R. Vleggaar, pp. 1-18. Elsevier Scientific Pub. Co., Amsterdam, The Netherlands.
Horwitz, W., Albert, R., and Nesheim, S. 1993. Reliability of mycotoxin assays: An update. J. AOAC Intl. 76: 461–491.
Ikebuchi, H., Teshima, R., Hirai, K., Sato, M., Ichinoe, M., and Terao, T. 1990. Production and characterization of monoclonal antibodies to nivalenol tetraacetate and their application to enzyme-linked immunoassay of nivalenol. Biol. Chem. Hoppe-Seyler 371(1): 31–36.
Kawamura, O., Sato, S., Kaji, H., Nagayama, S., Ohtani, K., Chiba, J., and Ueno, Y. 1989. A sensitive ELISA of ochratoxin A based on monoclonal antibodies. Toxicon 27: 887–898.
Kawamura, D., Sato, S, Nagura, M., Kishimoto, S., Ueno, I., Sato, S., Uda, T., Ito, Y., and Ueno, Y. 1990. ELISA for detection and survey of ochratoxin in live stock sera and mixed feeds. Food Agric. Immunol. 2(3): 135–144.
Kemp, H.A., Mills, E.W.C., and Morgan, M.R.A. 1986. Enzyme-linked immunosorbent assay of 3-acetyldeoxynivalenol applied to rice. J. Sci. Food Agric. 37: 888–894.
Koeltzow, D. and Tanner, S. 1990. Comparative evaluation of commercially available aflatoxin test methods. J. Assn. Offic. Anal. Chem. 73: 584–589.
Langone, J.J. and Van Vunakis, H. 1976. Aflatoxin B_1 specific antibodies and their use in radioimmunoassay. J. Natl. Cancer Inst. 56: 591–595.
Lee, S. and Chu, F.S. 1981a. Radioimmunoassay of T-2 toxin in corn and wheat J. Assn. Offic. Anal. Chem. 64: 156–161.
Lee, S. and Chu, F.S. 1981b. Radioimmunoassay of T-2 toxin in biological fluids. J. Assn. Offic. Anal. Chem. 64: 684–688.
Liu, M.T., Ram, B.P., Hart, L.P., and Pestka, J.J. 1985. Indirect enzyme-linked immunoassay for the mycotoxin zearalenone. Appl. Environ. Microbiol. 50: 332–336.
Mannon, J. and Johnson, E. 1985. Fungi down on the farm. New Scientist 105(1446): 12–16.
Martlbauer, E., Gareis, M., and Terplan, G. 1988. Enzyme immunoassay for the macrocyclic trichothecene roridin A: Production, properties and use of rabbit antibodies. Appl. Environ. Microbiol. 54: 225–230.
Mills, E.N.C., Johnston, J.M., Kemp, H.A., and Morgan, M.R.A. 1988. An enzyme-linked immunosorbent assay of diacetoxiscirpenol applied to the analysis of wheat. J. Sci. Food Agric. 42: 225–233.
Mills, E.N.C., Alcick, S.M., Lee, H.A., and Morgan, M.R.A. 1990. An ELISA for deoxynivalenol in wheat utilizing novel hapten derivation procedures. Food Agric. Immunol. 2(3): 109–118.
Morgan, M.R.A., McNerney, R., and Chan, H.W.S. 1983. Enzyme-linked immunosorbent assay of ochratoxin A in barley. J. Assn. Offic. Anal. Chem. 66: 1481–1484.
Morgan, M.R.A., McNerney, R., Chan, H.W.S., and Anderson, P.H. 1986a. Ochratoxin A in pig kidney determined by enzyme-linked immunosorbent assay. J. Assn. Offic. Anal. Chem. 37: 475–480.
Morgan, M.R.A., Kang, A.S., and Chan, H.W.S. 1986b. Production of antisera against sterigmatocystin hemiacetal and its potential for use in an enzyme-linked immunosorbent assay for sterigmatocystin in barley. J. Sci. Food Agric. 37: 873–880.
Morgan, M.R.A., Kang, A.S., and Chan, H.W.S. 1986c. Aflatoxin determination in peanut butter by enzyme-linked immunosorbent assay. J. Sci. Food Agric. 37: 908–914.
Mortimer, D.N., Shepherd, M.J., Gilbert, J., and Morgan, M.R.A. 1988. A survey of the occurrence of aflatoxin B_1 in peanut butters by enzyme-linked immunosorbent assay. Food Addit. Contam. 5: 127–132.
Nieuwenhof, F.F.J., Hoolwerf, J.D., and Van-Den-Beden, J.W. 1990. Evaluation of an enzyme immunoassay for the detection of aflatoxin M1 in milk using antibody-coated polystyrene beads. Milchwissenschaft 45: 584–588.
Park, D.L., Miller, B.M., Hart, P., Yang, G., McVey, J., Page, S.W., Pestka, J., and Brown, L.H. 1989a. Enzyme-linked immunosorbent assay for screening aflatoxin in cottonseed products and mixed feed: Collaborative study. J. Assn. Offic. Anal. Chem. 72: 326–332.
Park, D.L., Miller, B.M., Nesheim, S., Trucksess, M.W., Vekich, A., Bidigare, B., McVey, J.L., and Brown, L.H. 1989b. Visual and semiquantitative spectrophotometric screening method for aflatoxin B in corn and peanut products: Follow-up study. J. Assn. Offic. Anal. Chem. 72: 638–643.
Patey, A.L., Sharman, M., and Gilbert, J. 1992. Determination of total aflatoxin levels in peanut butter by enzyme-linked immunosorbent assay: Collaborative study. J. AOAC Intl. 75: 693–697.
Pestka, J.J. 1988. Enhanced surveillance of food borne mycotoxins by immunochemical assay. J. Assn. Offic. Anal. Chem. 71: 1075–1081.
Pestka, J.J. 1991. High performance thin layer chromatography ELISAGRAM: Application of a multi-hapten immunoassay to analysis of the zearalenone and aflatoxin mycotoxin families. J. Immunol. Methodol. 136: 177–183.
Pestka, J.J. and Casale, W.L. 1990. Naturally occurring fungal toxins. In "Food Contamination from Environmental Sources," ed. M.S. Simmons and J. Nriagu, pp. 613-638. John Wiley and Sons Ltd., New York.
Pestka, J.J. and Chu, F.S. 1984. Enzyme-linked immunosorbent assay of mycotoxins using nylon beads and Terasaki plate solid phases. J. Food Protect. 47: 305–308.
Pestka, J.J., Gaur, P.K., and Chu, F.S. 1980. Quantitation of aflatoxin B_1 and aflatoxin B_1 antibody by an enzyme-linked immunosorbent microassay. Appl. Environ. Microbiol. 40: 1027–1031.
Pestka, J.J., Li, Y., Harder, W.O., and Chu, F.S. 1981a. Comparison of radioimmunoassay and enzyme-linked immunosorbent assay for determining aflatoxin M_1 in milk. J. Assn. Offic. Anal. Chem. 64: 294–301.
Pestka, J.J., Lee, S.S., Lau, H.P., and Chu, F.S. 1981a. Enzyme-linked immunosorbent assay for T-2 toxin. J. Am. Oil Chem. Soc. 58: 940A–944A.
Pestka, J.J., Li, Y.K., Harder, W.O., and Chu, F.S. 1981b. Comparison of radioimmunoassay and enzyme-linked immunosorbent assay for determining aflatoxin M_1 in milk. J. Assn. Offic. Anal. Chem. 64: 294–301.
Ram, B.P., Hart, L.P., Cole, R.J., and Pestka, J.J. 1986a. A research note: Application of ELISA to retail survey of aflatoxin B_1 in peanut butter. J. Food Protect. 49: 792–795.
Ram, B.P., Hart, L.P., Shotwell, O.L., and Pestka, J.J. 1986b. Analysis of aflatoxin B_1 in naturally contaminated corn and cottonseed by enzyme-linked immunosorbent assay: Comparison with thin layer chromatography and high performance liquid chromatography. J. Assn. Offic. Anal. Chem. 69: 904–907.
Ramakrishna, N., Lacey, J., Candlish, A.A.G., Smith, J.E., and Goodbrand, I.A. 1990. Monoclonal antibody-based enzyme linked immunosorbent assay of aflatoxin B_1, T-2 toxin, and ochratoxin A in barley. J. Assn. Offic. Anal. Chem. 73: 71–76.
Sato, S., Nagayama, S., Kawamura, O., and Ueno, Y. 1987. Detection of ochratoxin A in meat, wheat, and plasma by an enzyme-linked immunosorbent assay. Proc. Japan. Assn. Mycotoxicol. 26: 47–50.
Shelby, R.A. and Kelley, V.C. 1992. Detection of ergot alkaloids from *Claviceps* spp. in agricultural products by competitive ELISA using a monoclonal antibody. J. Agric. Food Chem. 40: 1090–1092.
Trucksess, M.W., Stack, M.E., Nesheim, S., Park, D.L., and Pohland, A.E. 1989. Enzyme-linked immunosorbent assay of aflatoxins B_1, B_2, and G_1 in corn, cottonseed, peanuts, peanut butter, and poultry feed: Collaborative study. J. Assn. Offic. Anal. Chem. 72: 957–962.
Trucksess, M.W., Stack, M.E., Nesheim, S., Page, S.W., and Albert, R.H. 1991. Immunoaffinity column coupled with solution fluorometry or liquid chromatography post-column derivatization for determination of aflatoxins in corn, peanuts, and peanut butter: Collaborative study. J. Assn. Offic. Anal. Chem. 74: 81–88.
Warner, R. and Pestka, J.J. 1986. Screening for zearalenone in corn by competitive direct enzyme-linked immunosorbent assay. J. Agric. Food Chem. 34: 714–717.
Warner, R. and Pestka, J.J. 1987. ELISA survey of retail grain-based food products for zearalenone and aflatoxin B_1. J. Food Protect. 50: 502–503.
Wei, R.D. and Chu, F.S. 1988. Production and characterization of antibody against PR toxin. J. Food Protect. 51: 463–466.
Xu, Y.C., Zhang, G.S., and Chu, F.S. 1986. Radioimmunoassay of deoxynivalenol in wheat and corn. J. Assn. Offic. Anal. Chem. 69: 967–969.
Yu, J. and Chu, F.S. 1991. Immunochromatography of fusarochromanone mycotoxins. J. Assn. Offic. Anal. Chem. 74: 655–660.

Updated November 1994 from a paper presented during the IFT Toxicology and Safety Evaluation Division symposium, "Immunoassay Applications to Food Analysis," at the Annual Meeting of the Institute of Food Technologists, Chicago, Ill., July 10–14, 1993.

—Edited by Neil H. Mermelstein, Senior Associate Editor

X. RELATED PROCEEDINGS APPENDIX

None.

McDONNELL BOEHNEN
HULBERT & BERGHOFF LLP
300 SOUTH WACKER DRIVE
CHICAGO, ILLINOIS 60606
TELEPHONE (312) 913-0001